#24 attach

CGNE-62-1(1)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| | |
|---|---|
| In re the Application of | |
| Comai, et al. | Examiner: P. Moody |
| Serial No. 07/985,742 | Art Unit: 1804 |
| Filed: December 4, 1992 | DECLARATION UNDER 37 CFR 1.131 |
| For: FIGWORT PLANT PROMOTER AND USES | |

Honorable Commissioner of
Patents and Trademarks
Washington, DC 20231

Dear Sir:

We, Luca Comai, Margaret P. Sanger and Stephen Daniel Daubert do hereby declare as follows:

1. We are the inventors of the subject application. The work represented in the attached notebook pages was conducted in reference to the above-identified patent application in the United States at least prior to November 13, 1988.

2. Figwort mosaic virus 34S promoter construct pFWP-101 is described in the subject patent application.

3. Photocopies of relevant pages from Margaret P. Sanger's experimental notebook are attached hereto as Exhibit A.

4. Electroporation of plant protoplast with pFWP-101 was conducted as shown on notebook pages 67-69 in Exhibit A.

5. Electroporated protoplasts were analyzed and GUS expression confirmed as demonstrated on notebook pages 69-71 in Exhibit A.

DECLARATION

We declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 8/20/93 By: Luca Comai
Luca Comai

Date: 8/23/93 By: Margaret Sanger
Margaret P. Sanger

Date: 8/24/93 By: Stephen Daniel Daubert
Stephen Daniel Daubert

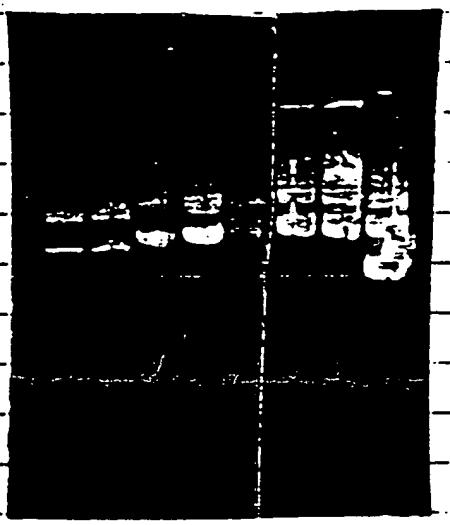
enclosures: Exhibit A

Next need to prepare enough DNA to test promoter region by transient expression system using GUS activity

inoculated 50ml LB (Cm Km/ml) with pCGN7307-101

- harvested - 7304-101 (50ml) & extracted plasmid DNA
- Meanwhile to Sal I cut FMV-X2 & FMV-X4
  - phenol/chloroform extracted
  - EtOH ppted
  - dried & resuspended in 20ul to test restriction digest on Datura stramonium

① FMV (Sal I cut) X2 - 1ul
② FMV X4 (Sal I cut) - 1ul
③ pCGN 7304-101 uncut - 1ul
④ pCGN 860 (35S-HM-tml3') 0.5ul
⑤ pCGN 852 (35S-HM-tml3') 0.5ul
⑥ pCGN 844 (nos3'-HL-Ocs3') 0.5ul
⑦ 7304 (A) - 1ul
⑧ 7304 (B) - 1ul



Checked concentration via A260

CGN 7304-101 250 x dil = .273 --- 2.3mg/ml
CGN 7304 250 x dil = .914 ---- 9.14mg/ml
CGN 3000 2500 x dil = .502 ---- 50mg/ml
CGN 860 1000 x dil = .532 ---- 13.3mg/ml
CGN 852 5000 x dil = .234 ---- 54.6mg/ml

Need to check CGN 3000 on gel

EXHIBIT A
USSN 07/985,742

- Electroporation of FMV promoter Region-GUS into tobacco protoplasts:

- Want to test out FMV full length RNA promoter region - to see if altered DNA works
  - If GUS's cause problems?
    - Generally - is there any promoter activity

Will test the following constructs for comparison w/ FMV:

(A) CGN 7000 (mas 5' - GUS - mas 3') -- 50ug + 175ug pUC19 DNA
(may have combined into (A) by mistake - assay will tell.)
(B) CGN 7304 (double 35S - GUS - Mas 3' -- 50ug + 125ug pUC19
(C) CGN 7304-101 (FMV - GUS - mas 3') -- 50ug + 175 ug pUC19
(D) pUC19 - no GUS 225ug DNA.

The protocol used was that of Enbow Jones & is outlined in my lab book #5808-002-E, 59+60

In vitro:
- 20 Xanthi leaves / 30 mls Enzyme Solution
- (- 17 Xanthi leaves / 30 mls Enzyme "
  - ~~infiltrated at 300 mm Hg 10-40 min~~
- incubated in dark ~ 2 hrs → 1.5?
- The material was agitated by running up + down wide bore (2nd Expanded pipettes + then narrower (normal) bore Expanded pipettes
- strained through 5 cm filter
- protos were centrifuged out of Enzyme ~ 2 min + washed 2x (#2 setting IEC Clinical Centri for 4.5 min each spin)
- counted protos - counted 3 sections + averaged:

protoplast Count @ 416
⑥ 415 $\bar{x} = 453$ ($\times 10^4$/ml)
⑦ 529

∴ have 8mls at $4.5 \times 10^6$ protos/ml
$= 36 \times 10^6$ protos

If electroporate 9 samples at 3.5 million/ml
then need $9 * 3.5 = 31.5 \times 10^6$ protos

- collected, spun → suspended in ELECTROPORATION BUFFER
- Added DNA's ÷ → 1ml/sample
- Electroporated ~ 60msec
~ 1200 μFarads
~ 225 μC Total DNA/sample

Following electroporation 4ml 1ml samples were added to 10ml culture medium in plant culture petri dishes (25×100 mm)

- Put to incubate in dark @ 25°C for ~2days.

Checked proto's in 2m — Still looked O.K.

- Proto's - O.K. - no microbial contamin.
- protos still intact + looking O.K.

So - need to harvest, extract, + assay for GUS activity.

Collected proto's by pipetting them into 15ml screw-cap centrifuge tube, sunrise water + plate w/ 3mls wash buffer → mix + centrifuge for 8min at ½ full (~500 rpm) for 8min (note had to respin 1 sample because disturbed pellet + found 4min at ¾ full is also O.K.).

M-S

- pipetted off supernatant & resuspended protos in 1ml Extraction Buffer:

GUS Extraction Buffer*

50mM $Na_2HPO_4$ (pH=7.0 w/ $NaH_2PO_4$)
10mM BME (14.4M stock) = .7ml/l
10mM $Na_2EDTA$
0.1% Sodium Lauryl Sarcosine
0.1% Triton X-100
→ Plus 1uM PMSF mw=174.2 should use ~100uM ~ 10mg/ml

- put in sonic bath for 2 x 60sec & checked for protoplast breakage -- poor - tried sonic more still at least 50% intact
- polytroned each sample for ~ 5sec each
- most protos were lysed & rest looked ruptured.

- put into 1.5ml conical cent. tubes & spun for 4min in cold. Used supernatant as crude enzyme solution.

GUS Assay

- used 100ul enzyme prep
- add 400ul of Assay buffer - Extraction buffer w/ MUG 1.25mM (MW~440)
  - 500ul 100mM
  - 12.5ul 100mM MUG
  - → 10ml ext. Buff.
  - → at 37°

so final assay conc of 500ul vol = 1mM
for 10ml use 125ul of a 100mM MUG solution.

Incubate for various times

MUG = methylumbelliferone glucuronide
(4-methyl umbelliferyl β-D-glucuronide MW~440 - 5x.)

* Richard A Jefferson (1987) "Assaying Chimeric genes in Plants: the GUS gene fusion system." Plant Molec. Biol. Rep. Vol

## Stop Buffer

Stop reaction w/ 900ul of 0.2M $Na_2CO_3$ (21.2g/L)
– this stops rx as well as $\uparrow$ fluorescence of Methylumbelliferone

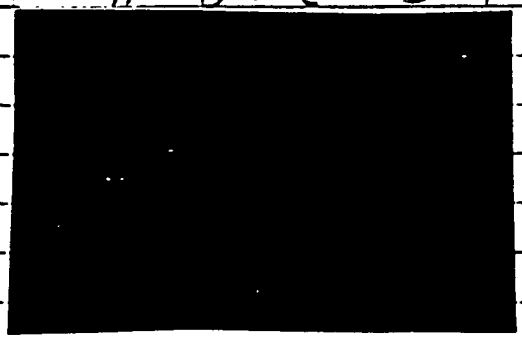
To correlate relative fluorescence with [MU] set-up standards (excit = 365, emiss = 455

Bubby recommends following conc range:

| | | | |
|---|---|---|---|
| 100 uM | = 0.1 uM | (5ul 10uM + 495 → 500) | 5 + 495 |
| 500 uM | 0.5 uM | (25ul 10uM + 475 EtBuf) | 25 + 495 |
| | 1.0 | (50ul 100uM + 495 EB) | 50 + 450 |
| | 2.0 | (100ul 100uM + 490) | 100 + 400 |
| | 4.0 | (200ul 100uM + 480) | 200 + 300 |
| | 8.0 | (400ul 100uM + 460) | 400 + 100 |

Added substrate to extracts at 3:30 PM
– took first time pt: @ 1hr 15min
– took 2nd time pt: @ 2hr 45min

1st looked at samples on UV transilluminator
(A) = CGN 7000 + CGN 7304 50+50+175 pc DNA
(B) = rinsed pipette tip of CGN 7304 (i.e. mainly no DNA) (175 plc)
(C) = CGN 7304-10 (50ul + 175 plcs)
(D) = pUC19 (225 ug)




This FMV-promoter region is active and may be 30 to a level similar to a single 35S of CaMV

Fluorometric assay → [illegible]

m.s